Non-leukemic pediatric mixed phenotype acute leukemia/lymphoma: genomic characterization and clinical outcome in a prospective trial for pediatric lymphoblastic lymphoma

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Abstract

Rare cases of hematological precursor neoplasms fulfill the diagnostic criteria of mixed phenotype acute leukemia (MPAL), characterized by expression patterns of at least two hematopoietic lineages, for which a highly aggressive behavior was reported. We present a series of 11 pediatric non-leukemic MPAL identified among 146 precursor lymphoblastic lymphomas included in the prospective trial Euro-LBL 02. Paraffin embedded biopsies of 10 cases were suitable for molecular analyses using OncoScan assay (n=7), fluorescence in situ hybridization (FISH) (n=7) or both (n=5). Except for one case with biallelic *KMT2A (MLL)* breaks, all cases analyzed by FISH lacked the most common translocations defining molecular subsets of lymphoblastic leukemia/lymphomas. Two non-leukemic B-myeloid MPALs showed the typical genomic profile of hyperdiploid precursor B-cell lymphoblastic leukemia with gains of chromosomes 4, 6, 10, 14, 18 and 21. One B-T MPAL showed typical aberrations of T-cell lymphoblastic lymphoma, such as copy number neutral loss of heterozygosity (CNN-LOH) at 9p targeting a 9p21.3 deletion of *CDKN2A* and 11q12.2-qter affecting the *ATM* gene. *ATM* was also mutated in a T-myeloid MPAL case with additional loss at 7q21.2-q36.3 and mutation of *NRAS*, two alterations common in myeloid disorders. No recurrent regions of CNN-LOH were observed. The outcome under treatment was good with all patients being alive in first complete remission after treatment according to a protocol for precursor lymphoblastic lymphoma (follow-up 3-10 years, median: 4.9 years). In summary, the present series of non-leukemic MPALs widely lacked recurrently reported translocations in lymphoid/myeloid neoplasias and showed heterogeneous spectrum of chromosomal imbalances.

KEYWORDS

mixed phenotype acute leukemia/lymphoma; copy number; pediatric lymphoblastic lymphoma; translocation

INTRODUCTION

Mixed phenotype acute leukemia/lymphoma (MPAL) are neoplasms with blasts that express characteristics of more than one hematopoietic cell lineage and are classified according to the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues (WHO)[1,2] as ambiguous lineage leukemias. The majority of MPAL present as leukemia, consequently, the guidelines for lineage determination and subtyping of precursor cell neoplasms were primarily established for flow cytometry methods.[3,4] Therefore, several of the current diagnostic criteria of the WHO classification are difficult to apply on tissue specimens. Nevertheless, lineage assignment according to the WHO criteria can be used with minor modifications for immunophenotyping in precursor neoplasms presenting as tissue manifestation.[5] MPAL can thus be diagnosed if lineage specific markers, i.e. CD3 for the T-cell lineage, myeloperoxidase for the myeloid lineage and/or two markers specific for the B-cell lineage like CD79a, CD19, PAX5 or CD22 are expressed in a precursor cell population simultaneously. By convention, the term lymphoma is used if the process is confined to a mass lesion with no or minimal evidence of peripheral blood and bone marrow involvement (<25% leukemic blasts).[1]

A variety of genetic lesions has been reported in the leukemic MPAL, some of which have led to the definition of distinct entities in the WHO classification characterized by recurrent molecular alterations. These include MPAL with the t(9;22)(q34;q11)/*BCR-ABL1* fusion and MPAL with t(v;11q23.3)/*KMT2A*-rearranged.[2] In addition, a specific type of acute myeloid leukemia (AML) with frequent tissue manifestation and co-expression of lymphoid markers, the AML with t(8;21)(q22;q22.1)/*RUNX1-RUNX1T1* is named as a separate entity. Other aberrations reported are del(6p), del(5q), 12p11.2 abnormalities, structural abnormalities of chromosome 7 and ploidy alterations.[6-8] Recent whole genome sequencing analyses have also identified t(6;14)(q25;q32) suggesting involvement of the *BCL11B* gene in two pediatric patients with T/Myeloid MPAL.[9] Since molecular and clinical studies of non-leukemic MPAL are lacking, it is still unknown whether the genetic and clinical features of this disease are comparable to leukemic MPAL or whether they are biologically closer to

lymphoblastic lymphoma. In fact, it is hitherto unclear how these mixed phenotype precursor neoplasias should be treated, i.e. whether a precursor-lymphoblastic lymphoma protocol is an adequate treatment option.

In 2011, we published a large series of 188 pediatric LBL treated within a prospective randomized trial (the Euro-LB 02 study), in which a detailed immunohistochemical analysis was performed.[5] Interestingly, out of 146 lymphoma cases with complete immunophenotypic data for lineage assessment according to the established guidelines, 11 (7%) displayed mixed phenotypes expressing markers of at least two hematopoietic lineages and were classified as MPAL/lymphoma according to the WHO guidelines. Most of these cases (64%) displayed a mixed B-myeloid phenotype. However, the genetic events associated with these cases were not defined. Thus, we have here performed the first molecular characterization of these non-leukemic pediatric MPAL and compared the results obtained to those reported for leukemic MPAL[10-16] and LBL/ALL.[17-19] Additionally, we documented the clinical outcome of these patients under treatment of the Euro-LBL trial for precursor lymphoblastic lymphomas.

MATERIALS AND METHODS

Study population

A total of 11 mixed lineage acute lymphomas, previously reported with regard to pathological features,[5] entered the study. Eight cases were male whereas three were female. The median age of the patients was 11 years (range 3-18). For all cases the diagnosis of mixed phenotype was made according to the WHO 2017 guidelines,[1] when the lymphoma cell population expressed specific markers of at least two lineages to a degree that it was not possible to assign the neoplasia to one lineage with certainty. Evidence of at least two B-cell markers was mandatory for determining B-cell lineage including CD19 in most cases, CD3 for the T-cell lineage and /or MPO for the myeloid lineage.

The study was performed in the framework of the clinical trial Euro-LB 02 (a treatment protocol for pediatric LBL patients run by the European Intergroup Cooperation on Childhood Non-Hodgkin Lymphoma as an International multicenter protocol), whose results have been

recently published.[20] The EURO-LB02 was performed after approval by the competent ethics committees and in accordance with an assurance filled with and approved by the departments of health and human services, where appropriate. The trial was registered at http://www.clinicaltrials.gov as #NCT00275106.

Immunochemistry and Fluorescence in situ hybridization (FISH)

Immunohistochemistry and FISH analyses were done on FFPE tissues as previously described.[5,21] FISH analyses were performed for the detection of breakpoints or gene fusions affecting the *IGH*, *KMT2A (MLL)*, *BCR*, *ABL1*, *ETV6*, *RUNX1* and *RUNX1T1* loci in nine cases with available material for molecular studies; from which seven cases were evaluable (cases 1-4 and 6-8). Moreover, a probe for the TCR beta (*TRB*) locus[22] was applied for the verification of 7q34 deletion (Probes are described in supporting Information Table 1). Evaluation of FISH was conducted according to standard procedures.[21] The digital image acquisition, processing, and evaluation were performed using ISIS digital image analysis system version 5.0 (Metasystems, Altlussheim, Germany).

Copy number analysis

DNA from 9 cases with available material for molecular studies was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue blocks using the phenol-chloroform extraction method as previously described.[23] DNA quality was tested using standard methods.[24] In 7 of the 9 cases, the DNA fulfilled the quality criteria for molecular analysis[24] (cases 3, 4 and 6-10). These were hybridized on the MIP-assay using the OncoScan FFPE Express custom service (Affymetrix, Santa Clara, USA). CN determination of the MIP assay has been previously described.[25] Gains, losses and regions of copy number neutral loss of heterozygosity (CNN-LOH) were defined by using Nexus 9.0 beta Discovery Edition software (Biodiscovery, El Segundo, CA) and evaluated by two different observers (IMG and IS)(Supporting Information).

Mutation analyses

Three somatic mutations in *MET, NRAS* and *ATM* genes detected by OncoScan were verified by direct sequencing using ABI PRISM 3100 Genetic Analyzer system (Applied

Biosystems, Foster City, CA). Details (including primer sequences) are described in the supporting material (Supporting Information and Supporting Information Table 2). Prediction of mutations impact was analyzed using SIFT (http://sift.jcvi.org/),[26] Polyphen http://genetics.bwh.harvard.edu/pph2/)[27] and CADD (Combined Annotation Dependent Depletion) (http://cadd.gs.washington.edu/home)[28] predictors (Supporting Information and supporting Information Table 3).

RESULTS AND DISCUSSION

Here, we present the clinical and molecular characteristics of 11 non-leukemic MPAL patients. The histomorphology in all cases showed a lymphoblastic cytology being indistinguishable from precursor lymphoblastic lymphoma. Granulated cytoplasms, maturation towards the myeloid lineage or Auer rods were not observed. The series included 7 lymphomas with B-cell and myeloid lineage markers, 2 with B-cell and T-cell lineage markers and 2 with T-cell and myeloid lineage markers. All cases were positive for TdT and 5 out of 10 available cases expressed CD34. In all but one case (# 8, Table 1), the expression of the different lineage markers seemed to be present in the majority of the blast population and thus could be named biphenotypic. It nevertheless remains difficult in tissue biopsies to exclude immunophenotypically separate populations in line with bilineage leukemia, without double staining, that was not performed in our cases. Six patients presented with a nodal manifestation and 5 had extranodal diseases (2 scalp/skin, 1 skin, 1 mediastinal, 1 testis) (Figure 1). The median age of the patients at diagnosis was 11 years (range 3-18 years) (Table 1). Seven cases showed stage 4, one stage 3 and three stage 2 disease. Flow cytometry was available for 4 of 7 patients with stage 4 disease due to bone marrow infiltration. The analyses are difficult to assess due to the low number of infiltrating blasts (Table 1) but confirmed the mixed phenotype in 2 cases in addition to the tissue based immunophenotypic results.

Out of the total of cases available for FISH analyses (n=9), seven finally fulfilled the quality criteria for being evaluated for breakpoints or gene fusions affecting the genes *IGH*, *KMT2A*, *BCR*, *ABL1*, *ETV6* and *RUNX1*. Case 3 carried a remarkably bi-allelic, *KMT2A* gene breakpoint (Figure 2A). The rest of cases (# 1, 2, 4, 6-8) lacked any of the recurrent breakpoints and translocations detectable with the applied probes (Table 1). The *BCR-ABL1* rearrangement and translocations involving *KMT2A* gene are the two genetic lesions most frequently reported in leukemic MPAL, the majority of which show B-cell and myeloid lineage. The frequency of *BCR-ABL1* and *KMT2A* translocations in pediatric B-myeloid MPAL has been reported to be 4-50%[10-14] and 11-25%,[6,9,14] respectively. However, these data should be interpreted with caution because in most of these studies the number of patients analyzed was very low (<25 cases). Surprisingly, in our series, only the case 3 with blasts meeting criteria for B-T lineage (a very unusual combination[1]), displayed *KMT2A* breaks. Three cases expressing CD34 (# 6, 7 and 8) were specifically screened for the translocation t(8;21)(q22;q22), all being negative (Supporting Information Table 4). Therefore, we excluded the diagnosis of tissue infiltration by a tissue manifestation/myelosarcoma of an acute myeloid leucemia with t(8;21)(q22;q22) in these cases. In a very recent genomic study on over 150 cases of pediatric mixed phenotype acute leukemias diagnosed by flow cytometry two new principal subgroups of MPAL were described, i.e. a T/myeloid and a B/myeloid subtype, the former with recurrent biallelic *WT1* alterations, the latter with recurrent *ZNF384* rearrangement.[29] The data reported by Alexander T.B. et al.[29] in addition, support a concept that founding molecular lesions in MPALs arise in early progenitor cells and prime these for lineage aberrancy and diversity, thus rendering MPAL a separable group from other lymphoblastic and/or myeloid neoplasias. Unfortunately, due to limited material in our tissue-based cases, we did not screen for these specific genomic alterations nor can comment on molecular evolution in our series. Moreover, the study by Alexander T.B. et al.[29] describes overlapping molecular features between the new provisional WHO-subtype of early-T-cell precursor acute lymphoblastic leukemia (WHO 2017) with the T/myeloid MPALs. Cases 8

and 11 of our cohort (Table 1) might be candidates for this group, nevertheless case 11 did not express CD34, a typical a marker of the early-T-cell precursor leukemias.[29]

CN profiling using the MIP assay method revealed chromosomal imbalances in all cases evaluable (n=7) (Figure 3A). Moreover, CNN-LOH was observed in five cases affecting the regions 9pter-p13.2 (2 cases), 11q12.2-qter, 20q12-qter and 21q21.2-q22.11 and chromosomes 15 and 16. The CNN-LOH detected at 11q12.2-qter was in line with FISH results in which biallelic breaks at *KMT2A* were detected (case 3). Two out of the four cases with B-myeloid lineage (# 6 and 7) showed chromosomal aneuploidies suggestive of high hyperdiploidy (HHD) (Figure 2B). These chromosomal aneuploidies included gains of chromosomes 4, 6, 10, 14, 18 and 21, like previously described HHD B-LBL and precursor B-ALL.[17] Such hyperdiploid profiles are known to be associated with very favorable prognosis in B-lineage ALL.[1] Apart from whole chromosome aneuploidies, several CN alterations were detected in the total series (mean alterations excluding aneuploidies: 3.14 alterations) with a total of six gains, nine losses, three homozygous losses and four high gains (Supporting Information Table 4). This complexity is in line with previous studies showing that MPAL cases have in general several chromosomal aberrations.[8,30] Remarkably, losses of 7pter-p15.2 and 7p12.3-p11.2 were observed in two cases (# 7 and 8). Moreover, homozygous loss of 9p21.3/*CDKN2A* was detected in two cases (# 3 and 9) (Figure 3B). Case 9 with B-myeloid lineage also showed homozygous loss of 9p24.1/*PTPRD*, a gene whose dysregulation has been proposed to be associated with leukemogenesis[31] and disruption of its tyrosine phosphatase domain has been related to lymphomagenesis in nodal marginal zone lymphoma.[32] Furthermore, 1/3 non-leukemic MPAL with T-lineage (case 3) resembled typical T-LBL since it displayed CNN-LOH at 9p targeted a deletion of 9p21.3/*CDKN2A* resulting in a homozygous deletion of the locus and CNN-LOH at 11q12.2-qter, including *CDKN2A* and *ATM*, respectively. The *ATM* gene was also found mutated in another case with T-lineage markers (case 8) (Supporting Information Figure 1). The mutation, located in exon 19 of *ATM* (c.2572T>C, p. F858L), has been frequently reported in breast cancer[33-35] and lymphoid malignancies,[36,37] although it has been also described to be present in healthy

people at very low frequency (MAF<0.05) (http://www.1000genomes.org/). The rate of 9p CNN-LOH observed in our series was slightly lower than in T-LBL cases (28.5% *vs* 47%),[38] where LOH at 9p has been suggested to be associated with good response to treatment and favorable outcome.[39] For the *ATM* locus, the rate of LOH in T-LBL patients has been described as less than 5% of the cases.[38] Additionally, another case with T-lineage (case 4) showed a mutation in the juxtamembrane domain of the *MET* gene (c.3029C>T, p.T1010I) (Supporting Information Figure 1), predicted to be deleterious by Polyphen and CADD algorithms (Supporting Information Table 3). This mutation has been previously associated with diverse types of cancer,[40-42] although it is also found in individuals without cancer, suggesting that T1010I is a rare polymorphism.[43] No deletions of 13q14.2 (*RB1*) or gain of 6q23.3 (*MYB*) (aberrations recurrent of T-LBLs[17]) were observed. Interestingly, case 8 (T-myeloid lineage), besides a 7q deletion (Supporting Information Figure 2) and the above described *ATM* mutation, showed a missense mutation in exon 1 of the *NRAS* gene (c. 38G>A, p.G13D)(Supporting Information Figure 1). This alteration has been reported to be one of the most common variations in *NRAS* gene,[44] occurring in 16% of acute myeloid,[45] 4% of myelomonocytic leukemia diseases in adolescents[46] and 2% of childhood acute lymphoblastic leukemia.[47] This mutation, predicted to de deleterious *in silico* (Supporting Information Table 3), has been associated with the loss of intrinsic GTPase activity and constitutive activation of the RAS protein.[48] A recent study has shown that constitutive RAS activation bypasses the requirement for a functional BCR/PI3Kδ axis to sustain lymphoma fitness.[49] In the present study, case 8 showed a similar outcome to those without mutations, so the clinical significance of this alteration in this type of tumor remains still unknown. No other abnormalities typical of myeloid lineage, such as 5q- or i(17q),[1,50] were found in our series. Interestingly, a recent whole-exome sequencing study in MPAL series including 5 pediatric cases involving myeloid lineage showed mutations in the *IKZF1, RUNX1, KRAS, NRAS, WT1* and *FLT3* genes in 4 out of 5 cases.[51] Hotspots of mutation in these genes (except *IKZF1)* were also interrogated in our series in the context of OncoScan FFPE Express custom design, finding mutations only in *NRAS*.

All patients were alive and free of disease at the last follow-up with a median follow up of 4.8 years. Although leukemic MPAL are regarded as a rather aggressive disease in adults and also behave more aggressively than ALL in children,[15,52] the outcomes of non-leukemic MPALs diagnosed within the context of the Euro–LB 02 were rather favorable, with no events observed among the analyzed patients.

In summary, we describe the molecular profile of a series of 11 non-leukemic MPAL. All cases showed complex karyotypes like leukemic MPAL but lacked previously reported primary aberrations. Most of the cases with B-myeloid phenotype shared secondary aberrations with both precursor B-LBL and precursor B-ALL. On the other hand, MPAL with evidence of T-lineage showed some typical aberrations resembling T-LBL. Moreover, one T-myeloid lineage case displayed a *NRAS* mutation and a 7q21-qter deletion, common in myeloid diseases. Nevertheless, we did not observe a characteristic molecular pattern that would allow us to describe non-leukemic MPAL as a distinct molecular entity. Our results remain limited by the small number of cases, which simply reflects the rarity of the disease. Certainly, larger scale molecular characterizations in leukemic MPAL allow much more insight into the biology and prognostic subgroups as recently demonstrated.[29] Nevertheless, the present study might influence oncological treatment decisions in the way that treatment by a chemotherapy protocol proven effective in precursor lymphoblastic lymphomas also seems to be effective in these mixed phenotype neoplasms. The molecular and clinical observations in our non-leukemic MPALs seem to support the most recently published data on childhood ambiguous lineage leukemias revealing a better outcome under lymphoid directed treatments compared to myeloid type primary treatment.[53]

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TABLE 1 Pathological and clinical features of 11 patients with non-leukemic MPAL cases

Case No.	Oschlies et al(4)	Age, Gender	Site	Lineages	T-lineage markers	B-lineage markers	Myeloid	TdT	CD34	Translocation*	Stage	BM	OS, Status
1	**1004**	3,m	Scalp/skin	B-myeloid	CD3:0	CD79a: 2, CD20:0, CD19:2 Pax5:2; μ:0;	MPO:2, CD68-	2	na	no	4	5'	10.1 y, alive in CR
2	**1123**	12,f	Cervical LN	B-myeloid	All (CD3 CD7, CD2):0	CD79a:2, CD20: 2, Pax:2, CD19:2	MPO:1, CD68-	2	0	no	4	6**	6.6 y, alive in CR
3	**1128**	14,f	Cervical LN	B-T	CD3+, CD2+, CD5+, CD7+, CD4-, CD8-	CD79a:2, CD20: 0, Pax:1, CD19:na	MPO:1, CD33:0, CD68-	2	0	*KMT2A* break (biallelic)	4	10	2.9 y, alive in CR
4	**1181**	18,m	Cervical LN	B-T	CD3+, CD2+, CD5+, CD4-, CD8-	CD79a:2, CD19:2, CD20:0, Pax: 0	MPO:0	2	0	no	4	7**	4.9 y, alive in CR
5	**6011**	14,m	Skin	B-myeloid	CD3:0	CD79a:2, CD19:2 Pax:2, CD20:1	MPO:2	2	0	ne	2	-	6.2 y, alive in CR
6	**6012**	9,f	Inguinal LN	B-myeloid	CD3:0	CD79a:2, CD19:2 Pax:2, CD20:1	MPO:2	2	1	no	2	-	4.7 y, alive in CR
7	**6060**	13, m	Inguinal LN	B-myeloid	CD3:0	CD79a:1, Pax:2, CD20:1, CD19:2	MPO:1	1	2	no	4	na	3.7 y, alive in CR
8	**6061**	7,m	LN	T-myeloid	CD3+, CD5+, CD2+, CD4+, CD1a-	CD79a:1, CD19:0 Pax:0, CD20:0	subpopul MPO:2	1	2	no	3	-	3.6 y, alive in CR
9	**6062**	11, m	Testis	B-myeloid	CD3:0	CD79a:2, CD10:2 Pax:na, CD20:1	MPO:2	2	2	ne	2	-	3.5 y, alive in CR
10	**6027**	5, m	Scalp/skin	B-myeloid	CD3:0	CD79a:1, Pax:2, CD20:1	MPO:2	2	2	na	4	na	5.4 y, alive in CR
11	**1032**	10, m	Mediastinal	T-myeloid	CD3:2, CD1a-	CD79a:0 complete	MPO: 1	2	0	na	4	15'	9.0 y, alive in CR

m, male; f, female; LN, lymph node; subpopul, subpopulation; 0 indicates negative, 1 indicates weak (<30% of positive tumor cells) and 2 indicates positive (>30% of positive tumor cells or weak staining in all tumor cells); ne, not evaluable; na, not available; y, years; OS, overall survival; CR, complete response; BM%, percentage of bone marrow blasts *Breakpoints affecting *IGH*, *KMT2A*, *BCR*, *ABL1*, *ETV6*, *RUNX1* genes were analyzed **marker evaluated by flow cytometry in the bone marrow confirmed mixed lineage phenotype 'marker evaluated by flow cytometry in the bone marrow confirmed only one lineage

FIGURE LEGENDS

Figure 1 Dermal infiltration of the skin/scalp of a 3-year-old male with non-leukemic MPAL (case 1). There is a dense diffuse infiltration of the dermal skin by lymphoblasts (A: H&E x 50 and B: H&E x 400). The immunophenotype shows expression of markers of the B-cell lineage like CD79a (C: CD79a x 400) and the myeloid lineage (E, MPO x 400) along with TdT (F: TdT x 400) whilst the marker for the T-cell lineage CD3 (D: CD3 x 400) remains negative.

Figure 2 A, FISH analysis of *KMT2A* rearrangement in case 3, using the commercial probe Vysis LSI MLL Dual Color, Break Apart Rearrangement Probe (Abbott, Lake Bluff, IL). Red and green arrows indicate cells with *KMT2A* breaks while yellow arrows display normal cells. **B,** Raw profile of CN results in 3 B-myeloid MPAL cases (# 6, 7 and 9) using Nexus 6.0 beta Discovery Edition. Proportion of gains and losses are shown from 1pter to 22qter on the X axis.

Figure 3 A, Copy number (CN) profiles of 7 non-leukemic MPAL cases including whole genome aneuploidies using Nexus 9.0 beta Discovery Edition. On the X-axis, the chromosomes are represented horizontally from 1 to Y, in the upper part of the graphic the Y-axis represents the percentage of cases showing the CN alterations. Gains are represented on the positive Y-axis and are colored in blue, whereas losses are represented in the negative Y-axis in red. **B,** Chromosome 9 view of the three cases (# 3, 5 and 9) showing losses in 9p and/or CNN-LOH.